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WITNESS my hand this
Ninth day of November 2004

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PROVISIONAL SPECIFICATION

Invention Title: A BIDENTATE MOTIF AND METHODS OF USE

Applicant: MEDVET SCIENCE PTY LTD

The invention is described in the following statement:

A BIDENTATE MOTIF AND METHODS OF USE

FIELD OF INVENTION

The present invention relates to a bidentate motif and use of the motif in methods of regulating cellular activities. The invention also includes methods of diagnosis of conditions relating to these cellular activities.

BACKGROUND

Although many cytokines such as IL-3, GM-CSF and IL-5 and growth factors such as PDGF and IGF-1 were initially discovered as mitogens by virtue of their ability to promote cell proliferation, many of these factors were later also found to be potent regulators of cell survival through their ability to suppress programmed cell death or apoptosis. These biological activities are regulated by the binding of the cytokine or growth factor to their cognate cell surface receptor which initiates an ordered series of signalling events that includes receptor dimerization, the activation of tyrosine kinases followed by the tyrosine phosphorylation of the receptor cytoplasmic tail, the binding of multiprotein signalling complexes to receptor phosphotyrosine residues via src-homology 2 (SH2) domains or phosphotyrosine-binding (PTB) domains and the activation of downstream signalling cascades that promote a cellular response. Although a large degree of redundancy has been encountered when attempting to ascribe specific signalling pathways to unique biological responses, one emerging concept is that cell proliferation and cell survival can be viewed as independent biological outcomes that are regulated by distinct, yet intimately entwined, signalling pathways.

Evidence that cell survival which is reliant on a variety of cellular activities, is regulated in an independent manner to cell proliferation comes from the identification of centralized signalling nodes that are professional regulators of cell survival in a variety of biological settings. One such example is the PI 3-kinase. The activation of PI 3-kinase has been observed in response to a wide range of cytokines and growth factors and leads to the generation of phosphatidyl inositol phosphates which in turn promote the activation of

pleckstrin homology domain proteins such as the serine-threonine kinase, Akt (or protein kinase B). Akt is able to regulate cell survival through the phosphorylation of selected downstream targets that modulate key aspects of cell viability such as gene transcription (IκB kinase, FKHR1), protein translation (mTOR), cell metabolism (GSK3b), and apoptosis (BAD). A number of cytokines and growth factors that are known to be potent regulators of cell survival such as interleukin-3 (IL-3), nerve growth factor (NGF), platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1) have been shown to regulate cell viability through PI 3-kinase signalling.

Despite the almost bewildering array of reports examining the regulation of intracellular signalling cascades by cytokines and growth factors, understanding the molecular mechanisms by which cell surface receptors initiate intracellular signalling pathways that specifically lead to cell survival and how these differ to those involved in regulating cell proliferation is largely unknown. Although there are many examples where specific tyrosine phosphorylation sites in cell surface receptors have been shown to be important in regulating cell proliferation, the receptor motifs involved in initiating cell survival signalling have proved far more elusive. Although there are clear instances where receptor tyrosine residues can be directly attributed to regulation of cell survival, there are many examples where receptor tyrosine phosphorylation cannot solely account for at least some of the biological activites of cytokines and growth factors. These results are suggestive of the possibility that some cell surface receptors may employ alternate novel receptor motifs for the regulation of cell survival.

It has been unclear until now how the binding of proteins to their receptors can result in the specialised functions associated with these molecules and their receptors. The signalling events which lead to the specialised functions are unknown. However various cellular proteins are implicated in the cascade of events culminating in the biological functions associated with various molecules. There are many ubiquitous proteins involved in cell signalling pathways and any one or more may be involved in relaying signals switched on by proteins binding to their receptor.

The 14-3-3 family of proteins is one such protein, which consists of 7 different isoforms and is expressed ubiquitously from yeast to humans. The ability of 14-3-3 to bind to a number of motifs in a wide range of signalling molecules suggests that 14-3-3 proteins may participate in a number of cell signalling pathways that may include mitogenesis, transformation and survival. Although 14-3-3 has been shown to bind a number of signalling molecules, it has been more difficult to determine how or where 14-3-3 can regulate signalling events directly or indirectly, or whether 14-3-3 is implicated at all.

Accordingly, an object of the present invention is to overcome some of the problems of the prior art and to understand how proteins can express their biological activities and to use this information to manipulate cellular functions.

SUMMARY OF THE INVENTION

In a first aspect of the present invention there is provided a bidentate motif capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell.

The present invention relates to a novel bidentate motif that is composed of two adjacent residues of tyrosine and serine which have been found to be involved in the binding of crucial cytoplasmic proteins which are involved in cell signalling pathways. In some cases, the cytoplasmic proteins are ubiquitous proteins involved in cell signalling pathways that may include mitogenesis, transformation and survival.

In a preferred embodiment, the present invention provides a bidentate motif capable of binding to at least one cytoplasmic protein, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic

protein can interact to activate cellular activity in the cell and wherein at least one of the tyrosine or serine residues will bind to the cytoplasmic protein.

In an even further preferred embodiment, the cytoplasmic proteins are Shc for tyrosine and 14-3-3 for serine such that the Shc interacts with the 14-3-3 which in turn activates a binding of a signalling molecule which then activates a cell signalling pathway. Accordingly, the present invention preferably provides a tyrosine/serine bidentate motif that is essential for cell survival and a convergence of phosphoserine and tyrosine signals through a novel Shc/14-3-3 axis.

In another aspect of the present invention, there is provided a bidentate motif capable of binding to a cytoplasmic protein comprising a tyrosine and a serine/threonine residue, said motif comprising the following sequence alignment:

$$N\text{-}X\text{-}X\text{-}\underline{Y}\text{-}\ (X)_{1\text{-}13}\text{-}[R/K/H/Q]\text{-}[X/\Psi]_{2\text{-}3}\text{-}\underline{S/T}\text{-}X\text{-}P$$

wherein X is any residue, Y is tyrosine, S/T is serine or threonine and Ψ is a hydrophibic residue or an equivalent thereof.

In yet another aspect of the present invention, there is provided a bidentate motif of a receptor capable of binding to a cytoplasmic protein comprising a tyrosine and a serine/threonine residue, said motif comprising the following sequence alignment:

$$\underline{Y}\text{-}\ (X)_{1\text{-}16}\text{-}[R/K/H/Q]\text{-}[X/\Psi]_{2\text{-}3}\text{-}\underline{S/T}\text{-}X\text{-}P$$

wherein X is any residue, Y is tyrosine, S/T is serine or threonine and Ψ is a hydrophibic residue or an equivalent thereof.

In another aspect of the present invention there is provided a method of modulating cellular activities in a cell, said method comprising

modifying phosphorylation of a Tyr and/or Ser residue of a bidentate motif capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the

residue and cytoplasmic protein can interact to activate cellular activity in the cell.

Preferably the residues are Tyr577 and Ser585 of the common βc of the GM-CSF/IL-5/IL-3 receptor.

In another aspect of the present invention, there is provided a method of activating cellular activities in a cell said method including:

inducing phosphorylation of a bidentate motif, a functional equivalent or analogue thereof capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell.and

subjecting the bidentate motif to a cytoplasmic protein to bind to the Tyr and/or Ser.

In yet another aspect of the present invention there is provided a method of modulating cellular activities in a cell, said method including:

modifying phosphorylation of a bidentate motif, a functional equivalent or analogue thereof capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell;

subjecting the bidentate motif to a cytoplasmic protein which binds to the tyrosine and serine residue; and

activating a cell signalling pathway by interacting the bound cytoplasmic protein with a signalling molecule involved in the pathway.

In another preferred aspect of the present invention, there is provided a method of inhibiting cell survival, said method including inhibiting the binding of a cytoplasmic protein to a bidentate motif capable of binding a cytoplasmic protein

and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell .

In another aspect of the invention, there is provided a method of inhibiting cell activation, said method including inhibiting the binding of a cytoplasmic protein to a bidentate motif, a functional equivalent or analogue thereof capable of binding a cytoplasmic protein which activates cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell.

In another aspect, there is also provided a method of treating a cytokine mediated condition in a cell said method comprising:

regulating the activation of phosphorylation of a bidentate motif, a functional equivalent or analogue thereof capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell.

FIGURES

Figure 1 shows the panel of constructs used to examine the role of Ser585 and Tyr577 residues in the ability of GM-CSF to regulate primary haematopoietic cell function.

Figure 2 shows the double motif encompassing Tyr577 and Ser585 is necessary and sufficient for GM-CSF-mediated stimulation of colony formation from hemopoietic cells.

Figure 3 shows the double mutant Y577F/S585G, but not the single mutants or F8 mutants, abolishes GM-CSF-stimulated cell survival.

Figure 4 shows the double mutant Y577F/S585G is not defective in STAT5, Akt, JAK2, or Erk activation.

Figure 5 shows Tyrosine 179 on 14-3-3 is critical for its association with Shc in response to GM-CSF stimulation.

STL-EN cells expressing the GM-CSFR were transfected with plasmids expressing wild type and mutant forms of 14-3-3-myc. 24hs after transfection the cells were starved for 18 hrs in medium with 0.5% FCS in the absence of cytokine. After starvation cells were stimulated with GM-CSF (50ng/ml) at 0, 5 and 15 minutes. Cells were then lysed and Shc immunoprecipitated using the anti-Shc antibody. Immunoprecipitates were subjected to immunoblot analysis using the anti myc antibody, the shc antibody. Tyrosine phosphorylation of the βc in response to GM-CSF was examined using the anti-phosphotyrosine monoclonal antibody 4G10 (Pβc). Lysates were also immunoblotted using βc antibodies to demonstrate equal loading.

Figure 6 shows Tyrosine 179 on 14-3-3 is critical for its association with PI 3-kinase in response to GM-CSF stimulation.

CTL-EN cells expressing the GM-CSFR were transfected with plasmids expressing wild type and mutant forms of 14-3-3-myc. 34 hrs after transfection the cells were starved for 18 hrs in RPMI medium containing 0.5% FCS in the absence of cytokine. After starvation cells were stimulated with GM-CSF (50ng/ml) for up to 5 minutes. Cells were then lysed and 14-3-3-myc was immunoprecipitated using a myc antibody and PI 3-k activity of the immunoprecipitates was measured. Shown are ^{32}P-labeled phosphatidylinositols (PIP) and the origin. (A). Quantification of the intensity of the ^{32}P-labeled phosphatidylinositols (PIP) is shown in B.

Figure 7 shows Tyrosine 179 of 14-3-3 is required for Akt but not Erk activation in response to GM-CSF.

A) CTL-EN cells expressing the GM-CSFR were transfected with plasmids expressing wild type and mutant forms of 14-3-3-myc and Akt-HA. 24hs after transfection the cells were starved for 24hs in medium with 0.5% FCS in the absence of cytokine. After starvation cells were stimulated with GM-CSF (50ng/ml) at 0, 5, 15 and 30 minutes. Cells were then lysed and cleared lysates were subjected to SDS-PAGE and immunoblotted sequentially using a cocktail of anti-phosphno-Akt-antibodies (Thr308 and Ser473), anti-HA antibody (12CA5) and anti-myc antibody (9E10). CTL-EN cells expressing the GM-CSFR were transfected with plasmids expressing wild type and mutant forms of 14-3-3-myc and Erk-HA. Cells were starved, stimulated with GM-CSF and lysed as described above. Lysates were subjected to SDS-PAGE and immunoblotted sequentially using an anti-phospho-Erk-antibody, anti-HA antibody (12CA5) and anti-myc antibody (9E10).

Figure 8 shows cells from leukaemia patients exhibit phosphorylation of Ser585 (CML, Panel A) or both Ser585 and Tyr577 (AML, Panel B) of the βc chain. White cells are extracted from peripheral blood by percol gradient centrifugation, cultured for 2 hours in 10% FCS and then treated with GM-CSF. The βc-chain was immunoprecipitated with 1C1 and 8E4 (anti βc-chain), and probed with GMB (anti-phospho-serine 585), 4G10 (anti-phospho-tyrosine) and 1C1/8E4.

Figure 9 shows the amino acid sequence of the common βc.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect of the present invention there is provided a bidentate motif capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residues and cytoplasmic protein can interact to activate cellular activity in the cell.

The present invention relates to a novel bidentate motif that is composed of two adjacent residues of tyrosine and serine which have been found to be involved in the binding of crucial cytoplasmic proteins which are involved in cell signalling pathways. In some cases, the cytoplasmic proteins are ubiquitous proteins involved in cell signalling pathways that may include mitogenesis, transformation and survival.

Many cytokines and growth factors have been shown to be potent regulators of cell survival and much effort has been devoted to mapping the intracellular signalling pathways leading to this essential biological response. However, little is known of the receptor motifs utilized by cell surface receptors to initiate the signals that promote cell survival.

The motif is bidentate by nature because of two critical amino acids, namely tyrosine and serine that are required to bind cytoplasmic proteins which will then preferably activate cascading effects of signalling systems within the cell.

The term "motif" as used herein, means a distinctiveamino acid sequence which is conserved and forms a unit in which the amino acids interact. .

Signalling molecules may be molecules involved in cellular pathways such as but not limited to those pathways involved in proliferation, survival or differentiation. Examples of such pathways may include the JAK/STAT pathway, the ras/MAP kinase pathway or the PI-3-Kinase pathway. All pathways may be involved directly or indirectly with these functions.

The term "cell signalling pathways" as used herein includes all cellular pathways and cellular reactions which contribute to the functioning of the cell. It is not restricted to reactions that arise from cytokine mediated binding to the receptor. However, it is most preferred that the activities are activated by cytokine binding.

The cytoplasmic protein will be appropriate for the amino acid, namely tyrosine or serine, however, it is preferred that the cytoplasmic proteins that bind to the amino cells are selected from the group including 14-3-3 protein, Shc, SHIP-2, WW-domain of the prolyl isomerase, Pin1 and the ubiquitin ligase, NEDD4 and any cytolplasmic protein capable of binding a further signalling molecule which activates a cascade of events preferably leading to cell signalling pathways or other pathways and biological functions in a cell such as mytogenesis, proliferation, transformation, differentiation and cell survival. More preferably, the cytoplasmic protein is 14-3-3 or Shc. Most preferably, the 14-3-3 will bind to serine and the Shc will bind to tyrosine.

The 14-3-3 protein is a family of proteins which consists of 7 different isoforms and is expressed ubiquitously from yeast to humans. The ability of 14-3-3 to bind to a number of motifs in a wide range of signalling molecules suggests that 14-3-3 proteins may participate in a number of cell signalling pathways that may include mitogenesis, transformation and survival. Although 14-3-3 has been shown to bind a number of signalling molecules, it has been more difficult to determine how 14-3-3 can regulate signalling events.

The term "signalling molecule" is any molecule that can signal a cell signalling pathway so as to cause an activation in the signalling pathway.

Shc will bind to Tyr via its PTB domain and has the potential to both positively and negatively regulate intracellular signalling. For example, in addition to its suggested positive role in promoting signalling via the Ras/Map kinase pathway through the recruitment of grb2/sos and via the PI 3-kinase pathway through the recruitment of a grb2/GAB2/PI 3-kinase complex, Shc is also known to recruit negative regulators of signalling including the phosphatases SHP2 and SHIP.

The cytoplasmic proteins which bind to the amino acid will in turn bind to further signalling molecules which can activate a cascade of events leading to cell signalling pathways and biological functions such as, but not limited to,

mitogenesis, proliferation, transformation, differentiation and cell survival or any other cytoplasmic molecule or protein which does not signal.

In a preferred embodiment, the present invention provides a bidentate motif capable of binding to at least one cytoplasmic protein, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell and wherein at least one of the tyrosine or serine residues will bind to the cytoplasmic protein.

The bidentate molecule of the present invention comprises two amino acid residues which are in close proximity to each other so as to provide suitable binding and interaction of cytoplasmic proteins which may in turn bind signalling molecules and mediate cell signalling pathways. It has now been found in the present invention that the cytoplasmic proteins that bind tyrosine and serine can interact with each other. This was evident when mutations of either one of the tyrosine or serine residues of the motif only partially effected cell survival.

In an even further preferred embodiment, the cytoplasmic proteins are Shc for tyrosine and 14-3-3 for serine such that the Shc interacts with the 14-3-3 which in turn activates a binding of a signalling molecule which then activates a cell signalling pathway. Accordingly, the present invention preferably provides a tyrosine/serine bidentate motif that is essential for cell survival and a convergence of phosphoserine and tyrosine signals through a novel Shc/14-3-3 axis.

Both amino acid residues of Tyr and Ser are available in the motif for binding. However, it is preferred that if only Tyr is bound by the cytoplasmic protein Shc, then via the Shc/14-3-3 axis, the 14-3-3 cytoplasmic protein can be activated to further activate a cell signalling pathway and subsequent biological functions. Similarly, it is further preferred that in the absence of Tyr binding to Shc, the 14-3-3 cytoplasmic protein can bind solely to the Ser585 residue and via the

Shc/14-3-3 axis, the 14-3-3 protein can be activated via the Shc(unbound) cytoplasmic protein.

In a further preferred embodiment, the induction of the Shc/14-3-3 axis is via a Tyr179 on the 14-3-3 cytoplasmic protein. Applicants have found that the Tyr179 is necessary for PI-3 kinase activation as well as 14-3-3 interaction with Shc. Moreover, AKT activation also results in response to cytokine binding preferably GM-CSF stimulation.

In another aspect of the present invention, there is provided a bidentate motif capable of binding to a cytoplasmic protein comprising a tyrosine and a serine/threonine residue, said motif comprising the following sequence alignment:

$$N\text{-}X\text{-}X\text{-}\underline{Y}\text{-}(X)_{1\text{-}13}\text{-}[R/K/H/Q]\text{-}[X/\Psi]_{2\text{-}3}\text{-}\underline{S/T}\text{-}X\text{-}P$$

wherein X is any residue, Y is tyrosine, S/T is serine or threonine and Ψ is a hydrophibic residue or an equivalent thereof.

In yet another aspect of the present invention, there is provided a bidentate motif capable of binding to a cytoplasmic protein comprising a tyrosine and a serine/threonine residue, said motif comprising the following sequence alignment:

$$\underline{Y}\text{-}(X)_{1\text{-}16}\text{-}[R/K/H/Q]\text{-}[X/\Psi]_{2\text{-}3}\text{-}\underline{S/T}\text{-}X\text{-}P$$

wherein X is any residue, Y is tyrosine, S/T is serine or threonine and Ψ is a hydrophibic residue or an equivalent thereof.

Preferably, the tyrosine and serine/threonine residue can react with cytoplasmic proteins and wherein the tyrosine and/or seine and their respective cytoplasmic proteins can interact to activate cellular activity in the cell.

More preferably, the cytoplasmic protein that binds to tyrosine and/serine are Shc, SHIP-2 and 14-3-3.

In a preferred embodiment, there is provided a binding motif of a receptor. The receptor may be any receptor that is capable of binding to an extracellular molecule or protein and which mediates its function through the binding of a cytoplasmic molecule or protein such as 14-3-3 protein or Shc, or any cytoplasmic molecule or protein capable of binding a further signalling molecule which activates a cascade of events leading to cell signalling pathways and biological functions such as mitogenesis, proliferation, transformation, differentiation and cell survival, or any other cytoplasmic molecule or protein which does not signal.

A receptor as used herein may be selected from the group including:

(1) GM-CSF/IL-3/IL-5 receptor
(2) IL6 human interleukin-6 receptor beta chain precursor (IL-6R-beta)
(3) LEPR human leptin receptor precursor (LEP-R) (OB RECEPTOR) (OB-R).
(4) TNR2 human tumor necrosis factor receptor 2 precursor (tumor necrosis factor
(5) VGR1 human vascular endothelial growth factor receptor 1 precursor
(6) TRK3 human receptor protein-tyrosine kinase TKT precursor (EC 2.7.1.112)
(7) Q01974 protein-tyrosine kinase transmembrane receptor ROR2 precursor
(8) FGR1 human basic fibroblast growth factor receptor 1 precursor (BFGF-R)
(9) Q15426 protein-tyrosine phosphatase, receptor-type, H precursor (EC 3.1.3.48)
(10) PTPM human protein-tyrosine phosphatase mu precursor (EC 3.1.3.48) (R-PTP-MU).
(11) PDGS human alpha platelet-derived growth factor receptor precursor (EC 2.7.1.112)
(12) FGR4 human fibroblast growth factor receptor 4 precursor (FGFR-4) (EC 2.7.1.112)

(13) FGR2 human fibroblast growth factor receptor 2 precursor (FGFR-2) (EC 2.7.1.112)

(14) Q13635 patched protein homolog (PTC)

(15) MANR human macrophage mannose receptor precursor.

(16) LRP2 human low-density lipoprotein receptor-related protein 2 precursor (megalin)

(17) IDD human integral membrane protein dgcr2/idd precursor (KIAA0163)

(18) AMFR human autocrine motility factor receptor precursor (AMF receptor) (gp78)

(19) ACH5 human neuronal acetylcholine receptor protein, alpha-5 chain precursor.

(20) KKIT human: stem cell growth factor receptor (proto-oncogene tyrosine-protein kinase kit) (C-KIT) (CD117 antigen)

(21) TPOR human: thrombopoietin receptor precusor (TPO-R) (myeloproliferative leukemia protein (C-MPL). TPOR or MPL.

(22) TPOR mouse: thrombopoietin receptor precursor (TPO-R) (myeloproliferative leukemia protein) (C-MPL). TPOR or MPL.

(23) Acetylcholine R

(24) Acetylcholine R alpha-5

(25) C-C chemokine receptor 6

(26) Middle T antigen

(27) integrin alpha 1

(28) FGFR2 (KGF R)

(29) FGFR1 (flg)

(30) FGFR5

(31) Erb4

(32) Vaccinia virus protein A36R

(33) Macrophage mannose R (MRC1)

(34) LDLR

(35) VLDL (rat)

(36) LRP1 low density lipoprotein receptor-related protein 1

(37) integrin beta 1

(38) interin beta 7

(39) integrin beta 3
(40) integrin beta 5
(41) integrin beta 6
(42) G-CSFR1 (second)
(43) G-CSFR1
(44) g-csf-r
(45) IL-6B (gp130)
(46) LeptinR
(47) ProlactinR
(48) insulinR
(49) irs-1
(50) IGFI R
(51) flt3 R
(52) VEGFR2 (FLK1)
(53) PDGF R-alpha
(54) IL-9R

or a functional equivalent or analogue thereof.

The receptor is preferably a cytokine receptor. More preferably it is the GM-CSF/IL-3/IL-5 receptor.

The binding capacity of the motif may be analysed by any binding studies or experiments available to the skilled addressee. Such experiments may include measuring the binding ability of a designated cytoplasmic protein to the binding motif. For instance electrophoretic mobility shift assays (EMSA or band shift assays) or foot print assays or pull down experiments are available to measure specific binding.

Hence the bidentate motif can be identified by the presence of a tyrosine or serine residue preferably in an amino acid sequence as described above, and the ability to bind a designated cytoplasmic protein. The designated cytoplasmic protein may be 14-3-3 protein, Shc or any cytoplasmic protein capable of binding a further signalling molecule which activates a cascade of

events leading to cell signalling pathways and biological functions such as mitogenesis, proliferation, transformation, differentiation and cell survival. More preferably, the cytoplasmic protein is 14-3-3 or Shc.

Preferably, the receptor is the GM-CSF/IL-3/IL-5 receptor which includes the common beta chain (β_c). It is found that the cytokines GM-CSF, IL-3 and IL-5 signal their actions through the surface receptor via the β_c. Most preferably, the binding motif comprises a sequence which includes amino acids Tyr and Ser corresponding to amino acids Tyr577 and Ser585 of the common β_c according to Figure 9 or a functional equivalent or analogue thereof.

The term "functional equivalent or analogue thereof" as used herein means a sequence which functions in a similar way but may have deletions, additions or substitutions that do not substantially change the activity or function of the sequence.

The common β chain (β_c) is a component of a cytokine receptor and is part of a signalling subunit of the receptor. It is thought that the cytokine signals its functions through the β_c, initiating events which cascade and culminate in an identifiable biological function such as cell survival, proliferation, differentiation and mature cell effector functions. However, the present invention is not limited to motifs of the β_c but includes motifs of receptors and other proteins within the cell having similar sequences to the β_c and including a serine/threonine residue. It is within the scope of this invention that the bidentate motif will have the structure identified above and through this structure, the cytokine may exert its effects on the cell via the bidentate motis and the Shc/14-3-3 axis. Preferably, the bidentate motif is found in the region of the βc which includes Tyr577 and Ser585. Having this as guide all proteins having a similar motif which corresponds to the region of βc including Tyr577 and Ser585 are within the scope of this invention which defines the bidentate motif.

The region or motif comprising amino acids Tyr577 and Ser585 of the common β_c or functional equivalent thereof may include the residues which preferably

interact with a cytoplasmic protein selected from the group including 14-3-3 protein, Shc, SHIP-2, WW-domain of the prolyl isomerase, Pin1, and the ubiquitin ligase, NEDD4 or any cytoplasmic protein capable of binding a further signalling molecule which activates a cascade of events leading to cell signalling pathways and biological functions such as mitogenesis, proliferation, transformation, differentiation and cell survival. However the present invention is not limited to this sequence but includes other equivalent sequences capable of performing the same function.

Throughout the description and claims of this specification, use of the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

In a preferred embodiment, the present invention provides a bidentate motif in the βc subunit of the GM-CSF, IL-3 and IL-5 receptors that is composed of two residues in close proximity to each other including Tyr577 and Ser585, which is preferably involved in the regulation of hemopoietic cell survival. Employing a panel of retroviral constructs for the expression of wt and mutant βc in primary hemopoietic cells derived from fetal livers it has now been found that this phosphotyrosine-phosphoserine bidentate motif is not only necessary for promoting cell survival and colony formation in response to GM-CSF, but that it is also sufficient for mediating these biological responses. This was evident from the inability of the βcTyr577Phe/Ser585Gly mutant to promote cell survival and colony formation in response to GM-CSF while conversely, the βcF7 mutant (in which Tyr577 and Ser585 remain intact while all 7 remaining tyrosine residues are substituted for phenylalanine) was able to promote cell survival and colony formation.

Any cell may have a bidentate motif. Accordingly, the bidentate motif may be present in any type of cell. The motif structure including the amino acid sequence as described above can be screened in any cell. However, preferably it is a cell that can be effected by GM-CSF or includes the common βc. Most preferably, the cell is one that is effected by binding of signalling

molecules to the common βc which harbours Tyr and Ser, more preferably corresponding to Tyr577 and Ser585 of the common βc. Most preferably, the cell is a haematopoietic cell such as, but not limited to, lymphoid, myeloid and erythroid cells. Specifically, the lymphoid lineage, comprising B cells and T cells, produces antibodies, regulates cellular immunity, and detects foreign agents such as disease-causing organisms in the blood. The myeloid lineage, which includes monocytes, granulocytes, and megakaryocytes, monitors the blood for foreign bodies, protects against neoplastic cells, scavenges foreign materials, and produces platelets. The erythroid lineage includes red blood cells, which carry oxygen. Accordingly, because the bidentate motif most preferably affects the haematopoietic cell lines, it is within the scope of the present invention that cellular activities associated with any of these cell lines may also be modulated by targeting a modification to Tyr577 and/or Ser585 of the common βc of GM-CSF/IL-5/IL-3.

Preferably the motif contains distinct tyrosine and serine motifs for the independent regulation of cell proliferation and cell survival. These independent biological responses was apparent in cells expressing the βcTyr577Phe/Ser585Gly mutant which were defective in their ability to survive in response to GM-CSF, but not in their ability to proliferate, while on the other hand cells expressing the βcF8 mutant were defective in their ability to proliferate in response to GM-CSF but not in their ability to survive.

In another embodiment of the present invention, it is preferred that the motif comprises a sequence selected from any one of the following sequences:

NGPYLG.....PP..HSRSLP
NVHYRT.....P...KTHTMP
**RYFTQKEE.....TESGSGP
NKKYELQDRDVCE....P.RYRSVSEP
NPTYSVM.....RSHSYP
NIFYLIR...KSGSFPMPELKLSISFP
NEEYLDLSQ.....PLEQYSPSYP
NQEYLDLSM.....PLDQYSPSFP
NATYKVD.....VIQRTRSKP
NPEY.....HSASSGP
NPDY.....WNHSLP
NPSYSSNPFVNYN....KTSICSKSNP

NTLY.....FNSQSSP
NPVYQKTTEDEVHI...CHNQDGYSYP
NPVYLKTTEEDLSIDIG..RH.SASVG
NPTYKMYEGGEPDDVGGLLDADFALDPDKPTNFTNPVY
NPIY.....KSAVTTVV
NPLY.....KSAITTTV
NPLY.....KEATSTFT
NPLY.....RKPISTHT
NPLY.....RGSTSTFK
PGHYL.....RCDSTQP
VQTYVLQ.....GDPRAVSTQP
QVLYGQLL.....GSPTSP
HSGYRHQVPSVQVF.....SRSESTQP
WKMYEVYDA.....KS.KSVSLP
KIPYFHA.....GGS.KCSTWP
ELDYCLKGLKL.....P.S.RTWSPP
SGDYMPM.....SPKSVSAP
SFYYSEENKLPEPEELDLEPENMESVP(LDPSASSSSLP)1283=survl.
EEIYIIM.....QSCWAFDSRKRPSFP
ISQYLQN.....S.KRKSRP
GTAY.....GLSRSQP
***YLPQEDWAP.....TSLTRP
LVAYIAFKRWNSCKQN...KQGANSRPVNQTPPPEGEKLHSDSGIS(phosphorylated)

The present invention has found that mutation of both Ser585 and Tyr577 is required to abolish hemopoietic cell survival in response to GM-CSF would suggest that these residues are able to independently regulate cell survival and that Tyr577 can compensate for the loss of Ser585 in the βcSer585Gly mutant while Ser585 can compensate for the loss of Tyr577 in the βcTyr577Phe mutant. Without being limited by theory, it is postulated that 14-3-3 is able to bind Ser585 and Shc is able to bind Tyr577 simultaneously on the same βc and that each adaptor or scaffold protein is able to regulate an intracellular signalling pathway that promotes cell survival. This bidentate configuration interacts to activate cellular activities

The 14-3-3 pathway regulated by Ser585 and the Shc pathway regulated by Tyr577 have the potential to signal through PI 3-kinase. Thus, the ability of Ser585 and Tyr577 to compensate for each other may be due to their respective ability to function as a bidenate scaffold that is able to regulate PI3-kinase through two alternate pathways.

The present invention therefore also provides an interaction between Shc and 14-3-3 such that the amino acid residues Tyr and Ser can exert their effects independently via the interaction between Shc and 14-3-3. Either Tyr or Ser activation (or phosphorylation) can activate Shc or 14-3-3 and cause the flow on effects to the signalling pathways via 14-3-3.

It is further found that Shc interacts with 14-3-3 via a Tyr 179 with is necessary for PI-3 kinase activity. Via this signalling molecule, further signalling pathways are activated leading to cellular activities such as mitogenesis, proliferation, transformation, differentiation, cell survival, chemotaxis, motility, enhanced phagocytosis, bacterial killing, superoxide production and cytotoxicity.

In another aspect of the present invention there is provided a method of modulating cellular activities in a cell, said method comprising

modifying phosphorylation of a Tyr and/or Ser residue of a bidentate motif capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell.

The modulation of the phosphorylation events which phosphorylate the tyrosine and/or serine residue on the bidentate motif will affect the binding of a cytoplasmic protein which in turn will affect the activation of signalling molecules which activate a cascade of events leading to cell signalling pathways and cellular activities. Preferably the cellular activities are selected from the group including mitogenesis, proliferation, transformation, differentiation, cell survival, chemotaxis, motility, enhanced phagocytosis, bacterial killing, superoxide production and cytotoxicity. Most preferably, the cellular activity is proliferation or cell survival. More preferably it is cell survival.

Therefore the modulation of the cellular activity is dependent upon the ability of the residues to bind to cytoplasmic proteins. The phosphorylating events are

critical for binding the cytoplasmic proteins to the tyrosine and/or serine. Both need not be phosphorylated nor bind to their respective cytoplamic proteins since the effect of the binding can be communicated via the Shc/14-3-3 axis. Either one of the cytoplasmic proteins needs to be activated to exert the effect via 14-3-3 which in turn can activate the PI-3 kinase through the Tyr179.

Hence the initiation of the regulating effects depend on the phosphorylation of the Tyr and/or Ser of the bidentate motif as described above.

"Modulation" or "Modulating" as used herein with respect to cellular activities means modifying or altering the activity compared to unmodified levels. The activity may be increased or decreased. For instance, proliferation may be increased or decreased. The modulation may cause an enhancement or reduction of the cellular activity.

The modification of phosphorylation of the Tyr or Ser may be an increase or a decrease of the phosphorylation of the residue. Methods of increasing or decreasing (inhibiting) phosphorylation are known to those skilled in the art. However, specifically, the use of specific kinase inhibitors are preferred to inhibit the phosphorylation.

In one preferred embodiment, the modification of phosphorylation is by inducing a mutation at the position of Tyrosine or Serine the common βc. More preferably the mutation is at a position equivalent to Tyr577 or Ser585 of the common βc. The mutation may include a substitution, deletion, or insertion of another amino acid such that the position of Tyr or Ser is debilitated.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements. "Conservative" amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include

alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

"Deletions" result from the amino acid being physically removed. The position may be targeted by methods available to the skilled addressee as used in site directed mutagenesis.

"Insertions" may arise where a similar amino acid is not inserted but another amino acid is inserted. Hence it is a non-conservative amino acid change.

Preferably, the substitutions replace Tyr or Ser, preferably Tyr577 or Ser585 of the common βc with another amino acid. Preferably the Tyr is replaced with phenylalanine, more preferably Tyr577Phe and the Ser is replaced by Gly, more preferably, Ser585Gly.

In another aspect of the present invention, there is provided a method of activating cellular activities in a cell said method including:

inducing phosphorylation of a bidentate motif, a functional equivalent or analogue thereof capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell.and

subjecting the bidentate motif to a cytoplasmic protein to bind to the Tyr and/or Ser.

Preferably the cytoplasmic protein is 14-3-3 protein or Shc, or any cytoplasmic protein capable of binding a further signalling molecule which activates a cascade of events leading to cell signalling pathways and biological functions such as mitogenesis, proliferation, transformation, differentiation cell survival,

chemotaxis, motility, enhanced phagocytosis, bacterial killing, superoxide production and cytotoxicity. Preferably the cytoplasmic binding protein is 14-3-3 or Shc.

The 14-3-3 or the Shc molecule binds not only to the bidentate motif via serine or tyrosine respectively, but has the ability to bind to a wide range of signalling molecules and to participate in a number of cell signalling pathways resulting in mitogenesis, transformation, differentiation cell survival, chemotaxis, motility, enhanced phagocytosis, bacterial killing, superoxide production and cytotoxicity.

Once 14-3-3, Shc or an equivalent binds to the bidentate motif, their ubiquitous nature can bind cytoplasmic proteins involved in signalling pathways which activate these pathways. The binding motif is phosphorylated and preferably phosphorylates the ^{585}Ser and/or ^{577}Tyr or equivalent residue. 14-3-3 and/or Shc can bind to the phosphorylated motif via these residues thereby positioning the 14-3-3 and the Shc close for further binding of cytoplasmic proteins involved in cell signalling (signalling molecules) for cellular activities such as proliferation, differentiation, cell survival, chemotaxis, motility, enhanced phagocytosis, bacterial killing, superoxide production and cytotoxicity.

The cell as used herein may be an isolated cell or be a cell contained within tissue or bodily fluids. Preferably the cell is a haematopoetic cell as herein described. Generally the cell will have a receptor for a cytokine such as the GM-CSF. Most preferably the cell will have the GM-CSF receptor and activate via the βc.

Inducing phosphorylation may include any means to phosphorylate the cell as described above. The cell may be directly subjected to phosphorylating agents. A kinase is preferred.

In yet another aspect of the present invention there is provided a method of modulating cellular activities in a cell, said method including:

modifying phosphorylation of a bidentate motif, a functional equivalent or analogue thereof capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell;

subjecting the bidentate motif to a cytoplasmic protein which binds to the tyrosine and serine residue; and

activating a cell signalling pathway by interacting the bound cytoplasmic protein with a signalling molecule involved in the pathway.

Preferably, the cytoplasmic protein is 14-3-3 protein, Shc or any cytoplasmic protein capable of binding a further signalling molecule which activates a cascade of events leading to cell signalling pathways and biological functions such as mitogenesis, proliferation, transformation, differentiation, cell survival, chemotaxis, motility, enhanced phagocytosis, bacterial killing, superoxide production and cytotoxicity. Preferably the cytoplasmic binding protein is 14-3-3 or Shc.

There are many signalling molecules involved in cellular pathways leading to cellular activity. However, it is preferred in the present invention to provide a molecule that binds to a phospho-serine bound 14-3-3 molecule or a phosphotyrosine bound Shc such that a pathway is coupled to the motif or equivalent unit in a receptor and brought into close proximity to downstream signalling proteins at, or near, the cell membrane. Cellular activities may include cell survival, proliferation, transformation, differentiation, mitogenesis, chemotaxis, motility, enhanced phagocytosis, bacterial killing, superoxide production and cytotoxicity.

Preferably the cellular activity is cell survival. Either one or both of the Tyr or Ser residues may be phosphorylated in the bidentate motif.

Phosphorylation of the motif may be modified by any means which results in inhibition or activation of the phosphorylation of the bidentate motif. Preferably, the Ser^{585} or the Try^{577} residue of the βc of a GM-CSF/IL-5/IL-3 is modified by phosphorylation.

For regulating cell survival, it is preferred to activate the PI-3-kinase pathway using a PI-3 kinase bound to a phosphoserine bound 14-3-3.

Regulation of cell survival may include enhancing or reducing cell survival or even causing cell death. This may be achieved by enhancing or inhibiting any of the steps described above. For instance enchancing phosphorylation of the bidentate motif may enhance survival. Alternatively, inhibiting phosphorylation may inhibit cell survival. Phosphorylation of one or the other of the Tyr or Ser is necessary for cell survival. Abolishing phosphorylation of both abolishes cell survival.

In another preferred aspect of the present invention, there is provided a method of inhibiting cell survival, said method including inhibiting the binding of a cytoplasmic protein to a bidentate motif capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell .

Preferably the cytoplasmic protein is 14-3-3 and/or Shc which bind to Ser or Tyr respectively. The binding may be inhibited in one or both.

Preferably the receptor is the GM-CSF/IL-5/IL-3 receptor although a phosphorylation event which phosphorylates ^{585}Ser or ^{577}Tyr of the common βc may also trigger the binding of 14-3-3 or Shc to the motif.

Inhibiting the binding of the cytoplasmic protein to the receptor may be achieved by inhibiting phosphorylation of the Tyr and/or Ser, mutating the Tyr and/or Ser or using antagonists.

Antagonists that bind to the bidentate motif wherein the motif is in either the phosphorylated or unphosphorylated form may be useful to inhibit cell survival or activation. Preferably antagonists may be useful to inhibit cell survival or activation by preventing phosphorylation preferably by preventing serine and/or tyrosine, preferably an equivalent Tyr577 or Ser585 of the common βc, phosphorylation of the β_c or equivalent thereby preventing the cytoplasmic protein binding to the bidentate motif. Alternatively, they may prevent the interaction of a signalling molecule binding to a phosphoserine bound 14-3-3 or phosphotyrosine Shc equivalent. Prevention of phosphorylation of the β_c or bidentate motif as described above may be by inhibition of the specific kinases involved in the phosphorylation of the serine/threonine or tyrosine residue or it may include mutation of the bidentate motif to prevent the cytoplasmic protein such as 14-3-3 or Shc from binding and activating cell cycle pathways. Kinase inhibitors such as H89 which binds to PKA may be used.

Antagonists may include antibodies, small peptides, small molecules, peptide mimetics or any type of molecule known to those skilled in the art that are directed to the bidentate motif so as to prevent attachment of cytoplasmic proteins such as 14-3-3 to a phosphoserine residue or a Shc to a phosphotyrosine residue or a signalling molecule. Antibodies may be generated in response to any of the bidentate motifs described above by methods known and available to the skilled addressee.

Hence, the antagonists as described may be useful as cancer therapeutics to prevent cell survival of cancer cells or cell activation such as myeloid cell activation and may be useful for preventing or treating leukaemia such as acute myeloid leukaemia (AML). Other uses of antagonists may be in prevention and treatment of inflammatory diseases.

This may be useful to prevent those functions related to cell activation, particularly myeloid cell activation. The functions may be selected from the group including chemotaxis, motility, enhanced phagocytosis, bacterial killing, superoxide production and cytoxicity. These functions may also contribute to inflammation including, but not limited to, asthma and rheumatoid arthritis.

In another aspect of the invention, there is provided a method of inhibiting cell activation, said method including inhibiting the binding of a cytoplasmic protein to a bidentate motif, a functional equivalent or analogue thereof capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell.

This method of interaction may be a useful tool for a method of treating or preventing cell proliferative diseases such as AML or cancer.

Inhibition may be by way of the use of antagonists as described above, or inhibition of phosphorylation of the bidentate motif or by any means that prevents activation of cell cycles via the bidentate motif described in the present invention.

It is also preferred to target the interaction between the cytoplasmic protein Shc and 14-3-3. It has been found that Shc interacts with 14-3-3 via a Tyr179 on the 14-3-3 molecule. Targeting or inhibiting this interaction may prevent the action of 14-3-3 to further activate and interact with triggering molecules such as PI-3 kinase which activates the PI-3 kinase pathway.

Also, it is preferred that where one or the other of Tyr or Ser is phosphorylated in the absence of the other, the cell will rely in the interaction between Shc and 14-3-3. Therefore by interfering with this interaction, further cell activations are interfered with.

In another aspect, there is also provided a method of treating a cytokine mediated condition in a cell said method comprising:

regulating the activation of phosphorylation of a bidentate motif, a functional equivalent or analogue thereof capable of binding a cytoplasmic protein and activating cellular activities in a cell, said bidentate motif comprising a tyrosine and a serine/threonine residue which are capable of interaction with cytoplasmic proteins, and wherein the residue and cytoplasmic protein can interact to activate cellular activity in the cell.

The cytokine mediated condition is a condition which requires a cytokine to bind to its receptor to induce a cellular activity. By regulating the activation, cellular activity may be activated to increase the phosphorylation or to decrease phosphorylation depending on the condition to be treated. Preferably, the cytokine mediated condition is a GM-CSF/IL-5/IL-3 mediated condition and the bidentate motif includes the amino acids ^{577}Tyr and ^{585}Ser of the common βc.

In the present invention it is shown that Tyr577 and Ser585 function to promote hemopoietic cell survival in response to GM-CSF. Ser585 and Tyr577 may also function independently of each other in a non-redundant manner. In terms of a specific role for Ser585, GM-CSF was able to promote colony formation, cell survival and cell proliferation in cells expressing the βcSer585Gly mutant in the presence of 10% FCS, a defect was found in cell survival when experiments were performed in low serum concentrations (i.e. 0.1% FCS). These results would suggest that under reduced serum conditions where the concentration of survival cytokines and growth factors is low, Ser585 plays an essential role in regulating hemopoietic cell survival in response to GM-CSF. Thus, it is possible that the Ser585 pathway is important in regulating GM-CSF-mediated hemopoietic cell survival *in vivo* under conditions where the concentrations of other cytokines and growth factors is limiting. Such conditions would presumably not include myeloid cells at sites of inflammation where the extracellular milleu is known to contain, in addition to GM-CSF, high concentrations of other cytokines and growth factors. However, myeloid cells such as monocytes/macrophages and dendritic cells are known to reside in

peripheral tissues not undergoing inflammation where they are thought to function as sentinels on guard against foreign pathogens. The long-term survival of these cells in the periphery where the concentrations of cytokines is likely to be more limiting may be dependent on the ability of GM-CSF to promote signalling via the Ser585 pathway.

In terms of a specific role for Tyr577, cells expressing the βcTyr577Phe mutant demonstrated increased colony formation and colony size in response to GM-CSF. In addition, this receptor mutant promotes increased hemopoietic cell survival. These findings using primary hemopoietic cells are the first to demonstrate a biological role for Tyr577 and would suggest that this residue plays a negative role in βc signalling. The adaptor protein Shc, which is known to bind Tyr577 via its PTB domain, has the potential to both positively and negatively regulate intracellular signalling. For example, in addition to its suggested positive role in promoting signalling via the Ras/Map kinase pathway through the recruitment of grb2/sos and via the PI 3-kinase pathway through the recruitment of a grb2/GAB2/PI 3-kinase complex, Shc is also known to recruit negative regulators of signalling including the phosphatases SHP2 and SHIP. Alternatively, Tyr577 may bind other PTB domain proteins that negatively regulate receptor function, however, the direct binding of other PTB domain proteins has not been reported.

Cell surface receptors with seemingly disparate biological functions are commonly observed to utilize similar strategies for the transduction of intracellular signals. For example, receptor tyrosine phosphorylation and the recruitment of SH2 and PTB domain proteins is a widely employed mechanism to physically couple activated receptors to downstream signalling pathways. Therefore, our finding that similar tyrosine/serine bidentate motifs occur in a wide variety of cell surface receptors would suggest that the bc may be a prototypic example of a novel signalling device employed for the regulation of specific biological responses to other cytokines and growth factors.

The present invention may also be used as a model for proliferative diseases. Given the interaction between the bidentate motif and the cytoplasmic proteins, any part of the interactions can be monitored to determine any aberration between the cells in question and that of a normal cell. Aspects of the model may analyse the phosphorylation ability of the Tyr or the Ser residues or analyse the interaction between the respective cytoplasmic proteins of Shc and 14-3-3 both with Tyr or Ser or between themselves.

A model may also be based on the activity of the 14-3-3 molecule and the activity or phosphorylation ability of the Tyr179 of the 14-3-3. Aberrations in any of these points may indicate a disorder in proliferation ability of the cell.

Preferably the model is based on a haematopoetic cell as described above. More preferably, the cell has a GM-CSF receptor including the Tyr577 and Ser585 of the common βc upon which the model is based.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Examples of the procedures used in the present invention will now be more fully described. It should be understood, however, that the following description is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Example 1: Role of Ser585 an βctyrosine residues in the ability of GM-CSF to regulate primary haematopoietic cell function.

a) Transduction of primary fetal liver cells

The ability of wt and mutant βc to transduce specific biological responses following GM-CSF stimulation was assessed in primary mouse hemopoietic cells derived from fetal livers. Fetal liver cells were harvested from E12.5 mice and transduced with bicistronic retroviral constructs for the expression of both the α subunit (GMRα) and the βc subunit of the human GM-CSF receptor. Human GM-CSF receptors were transducted into a β subunit null background using fetal liver cells derived from βc -/- β_{IL-3} -/- double knockout SV129 mice. For these transductions, ψ2 retroviral packaging cell lines were firstly stably transfected with constructs for the wild type GM-CSF receptor (pRUF-IRES-GMRαβc) and mutant receptors (pRUF-IRES-GMRαβcTyr577Phe, pRUF-IRES-GMRαβcSer585Gly, pRUF-IRES-GMRαβcSer585Gly/Tyr577Phe, pRUF-IRES-GMRαβcF8 and pRUF-IRES-GMRαβcF7. Pools of stably transfected viral-producing cell lines were then used to transduce primary hemopoietic cells derived from fetal livers. Transductions were set up by co-culturing irradiated (30Gy) ψ2 cells ($8x10^5$/ml) with fetal liver cells (** /ml) in IMDM, 15% heat inactivated fetal calf serum (HI FCS), 0.5ug/ml polybrene, 0.2 ng/ml mouse SCF. After 48 hours loosly adherent fetal liver cells were harvested by gently shaking and the cells transferred to a fresh flask overnight prior to use in functional assays.

b) Colony assays

The ability of GM-CSF to promote colony formation is dependent of the ability of the βc to promote two critical biological responses; cell survival and proliferation. The lack of colonies observed for the βcTyr577Phe/Ser585Gly and the βcF8 mutant could be due to a defect in their regulation of either cell survival, cell proliferation or both survival and proliferation. To examine these

individual possibilities, we examined the ability of the wt and mutant βc to specifically promote either cell survival or proliferation in response to GM-CSF. For the survival assays, fetal liver cells as prepared in Example 1 were transduced with wt and mutant βc and plated out in either no factor, 50ng/ml GM-CSF or control cocktail as a positive control for cell viability. After 24 or 48 hours, cells were double-stained firstly for GM-CSF receptor expression using the 4H1 anti-GMRα monoclonal antibody and an anti-mouse-PE antibody, and secondly for apoptosis using annexin-V-FITC. Cells expressing the GM-CSF receptor (4H1 positive) were examined for cell viability (annexin-V-FITC negative) by flow cytometry and the results are shown in Figure 3. While GM-CSF was able to promote the survival of fetal liver cells transduced with the wtβc, βcTyr577Phe, βcSer585Gly, βcF8 and the βcF7 mutants, survival of cells expressing the βcTyr577Phe/Ser585Gly was dramatically reduced. The combined observations that the βcTyr577Phe/Ser585Gly mutant was defective in promoting survival whilst the βcF7 mutant (where Tyr577 and Ser585 are intact) was able to promote cell survival would suggest that these residues constitute a distinct motif that is both necessary and sufficient for regulating hemopoietic cell survival. Furthermore, these results would indicate that the lack of colonies observed in Figure 2 for cells expressing the βcTyr577Phe/Ser585Gly mutant was due, at least in part, to the inability of this receptor mutant to transduce pro-survival signals in response to GM-CSF.

It is also noteworthy that while the βcF8 mutant was unable to promote colony formation in response to GM-CSF (Figure 2), it was not defective in mediating cell survival (Figure 3). This would imply that while tyrosine phosphorylation of βc is not important for regulating hemopoietic cell survival in response to GM-CSF, it may be important in regulating other signalling pathways necessary for colony formation. In addition, we have also consistently observed that the βcTyr577Phe mutant promotes increased survival even in the absence of GM-CSF. This data, together with findings in Example 1 that the βcTyr577Phe mutant promotes increased colony number (Figure 2) and colony size would suggest that Tyr577 of βc plays an important role in negatively regulating GM-

CSF signalling and that these signals are clearly distinct and dissectable from those generated by the Tyr577/Ser585 bidentate motif.

To examine the role of Ser585 and βc tyrosine residues in the ability of GM-CSF to regulate primary hemopoietic cell function we have generated a panel of bicistronic retroviral vectors that allow the co-expression of the human GM-CSF α subunit (GMRα) and either wild type (wt) or mutant common β subunit (βc) in primary mouse hemopoietic cells derived from fetal livers. This panel of βc mutants includes a Ser585Gly mutant which we have shown to be defective in 14-3-3 binding (pRUF-IRES-GMRαβcSer585Gly), a Tyr577Phe mutant which is shown to be defective in Shc binding (pRUF-IRES-GMRαβcTyr577Phe), a Ser585Gly/Tyr577Phe double mutant (pRUF-IRES-GMRαβcSer585Gly/Tyr577Phe), a mutant in which all 8 βc cytoplasmic tyrosines were substituted for phenylalanine (pRUF-IRES-GMRαβcF8), and an add back mutant in which Tyr577 and Ser585 remain intact while all 7 remaining cytoplasmic tyrosines were substituted for phenylalanine (pRUF-IRES-GMRαβcF7). For simplicity, these constructs will be referred to as wtβc, βcTyr577Phe, βcSer585Gly, βcTyr577/Ser585, βcF8 and βcF7 respectively (Figure 1).

These retroviral constructs were transduced into primary fetal liver cells derived from βc -/- βIL-3 -/- double knockout mice. The ability of the wt and mutant βc to promote colony formation in response to GM-CSF was examined. Transduced fetal liver cells were plated in soft agar in the presence of either GM-CSF or a positive control cytokine cocktail containing stem cell factor (SCF), IL-6 and G-CSF (control cocktail). After 2 weeks, colonies were counted and the results are shown in Figure 2. GM-CSF was able to promote colony formation in fetal liver cells transduced with the wtβc, βcTyr577Phe, βcSer585Gly and also the βcF7 add back mutant in which Tyr577 and Ser585 remain intact. However, colony formation in response to GM-CSF was essentially abolished in cells expressing either the βcF8 or the βcTyr577Phe/Ser585Gly mutants.

The inability of the βcTyr577Phe/Ser585Gly mutant to promote GM-CSF-mediated colony formation in Figure 2 indicates that these residues play an essential role in this biological response and that signals generated by Tyr577 and Ser585 cannot be compensated for by any of the remaining 7 βc tyrosine residues. Furthermore, these results define a minimal βc bidentate motif composed of Tyr577 and Ser585 that is not only necessary for promoting GM-CSF-mediated colony formation but is also sufficient for mediating this response as Tyr577 and Ser585 which remain intact in the βcF7 mutant were able to promote colony formation in response to GM-CSF to levels essentially equivalent to that observed for the wtβc.

During the course of these experiments it was consistently observed that not only did the βcTyr577Phe mutant promote increased colony number in response to GM-CSF when compared to cells expressing the wtβc (Figure 2A), but also colony size was significantly increased. These results suggest that Tyr577 and Ser585 may not only constitute a bidentate motif that is important for promoting colony formation, but that Tyr577 may also have an additional role independent of Ser585 that negatively regulates βc function. We considered the possibility that cells transduced with the βcTyr577Phe mutant were defective in a critical checkpoint in the GM-CSF-mediated differentiation program and that a delay in cellular senescence that normally accompanies terminal differentiation allowed these cells to continue proliferating leading to larger colonies. We therefore examined the hemopoietic cell lineage of the colonies obtained in response to GM-CSF from cells transduced with the wt and mutant βc using an *in situ* *tristain*. GM-CSF promoted almost exclusively monocyte/macrophage colonies in fetal liver cells transduced with the wtβc. No significant differences between the wtβc and the βcTyr577Phe mutant in terms of their ability to promote the formation of monocyte/macrophage colonies in response to GM-CSF was observed. Similarly, GM-CSF was able to promote monocyte/macrophage colony formation in fetal liver cells transduced with the βcTyr577Phe, βcSer585Gly and the βcF7 mutants indicating that none of these βc mutants were significantly affected in terms of differentiation. Furthermore,

no difference in the ability of GM-CSF to upregulate the expression of the F4/80 monocyte/macrophage marker in cells expressing either the wtβc or βcTyr577Phe mutant was observed (data not shown). Thus, the increased colony formation observed for fetal liver cells transduced with the βcTyr577Phe was not due to an overt defect or delay in the differentiation program induced by GM-CSF.

Example 2: The ability of the wt and mutant βc to specifically promote with cell survival or proliferation in response to GM-CSF

a) Annexin V staining

The ability of GM-CSF to promote cell survival was determined by annexin V-FLUOS (**) staining essentially as recommended by the manufacturer and as previously described (**). Transduced fetal liver cells were plated in IMDM and either 0.1% or 10% HI FCS, containing either no GM-CSF, 50ng/ml hGM-CSF or 1:1000 pro-survival cocktail (IL-6/EPO/G-CSF). After 48 hours, cells were harvested and stained firstly with the 4H1 anti-GMRα monoclonal antibody and an anti-mouse IgG-PE antibody and secondly wtih annexin V-FLUOS. Viable cells (annexin V-FLUOS-negative) expressing the GM-CSF receptor (GMRα-positive) were analysed by flow cytometry.

b) Colony assays

The response of immature cells to GM-CSF was analysed using colony formation assays. Fetal liver cells were transduced and expression of the GM-CSF receptor quantitated as described above. Colony forming cells were assayed using a double layer agar assay. Plates were prepared with underlayers comprised of IMDM supplemented 0.5% agar (Difco) containing cytokines as shown in the figures. An overlayer was added with transduced cells, at a concentration of 100,000 per dish, in 0.3% agar. All media contained 10% HI FCS (JRH Biosciences) with penicillin and streptomycin added.

All cytokines were diluted in PBS and a control of PBS alone was added in each assay to verify that colonies were formed in response to cytokine stimulation.

Additionally, all assays included a general stimulus composed of a cocktail of IL-6 (100 ng/ml), SCF (100 ng/ml) and erythropoietin (4U/ml).

Plates were incubated at 37°C 5% CO_2 for 14 days and colonies counted using an inverted microscope at the completion of this time.

Example 3: The ability of GM-CSF to promote the survival of fetal liver cells transduced with the βcSer585Gly mutant under low serum conditions.

While our previous studies had demonstrated a defect in GM-CSF-mediated survival for the βcSer585Gly mutant in the CTL-EN cell line, data presented here indicates that this mutant was not defective in promoting either colony formation (Figure 2) or survival (Figure 3A) of primary hemopoietic cells in response to GM-CSF. However, the experiments presented here with fetal liver cells were performed in the presence of 10% FCS which we have previously observed to mask the survival defects of some βc mutants. Fetal liver cells expressing either wtβc or βcSer585Gly were plated out in 0.1% FCS and either no factor, GM-CSF or control cocktail. Under these conditions, GM-CSF was able to promote the survival of fetal liver cells expressing the wtβc but not the βcSer585Gly mutant (Figure 3A). These results would indicate that the Ser585 survival pathway that was initially identified in CTL-EN cell lines is also important for regulating the survival of primary hemopoietic cells.

Example 4: The ability of GM-CSF to promote the proliferation of fetal liver cells transduced with either wt or mutant βc receptors.

(a) 5-Bromo-2'-deoxyuridine (BrdU) incorporation assays

The ability of GM-CSF to promote cell cycle progression was determined by BrdU incorporation using the in situ cell proliferation kit (Roche). Briefly, fetal liver cells transduced with either the wt or mutant GM-CSF receptors were firstly stained with the 4H1 anti-GMRα monoclonal antibody followed by anti-mouse IgG-PE and GM-CSF receptor-positive cells were purified using fluoresence activated cell sorting (FACS). Purified cells were then plated out in IMDM and 10% HI FCS containing either no GM-CSF, 50ng/ml hGM-CSF or control

cocktail for 24 hr with the cells being pulsed with BrdU (Roche) for the last four hours. Cells were then fixed and stained with anti-BrdU-FITC antibodies and BrdU incorporation was assessed by flow cytometry.

Transduced cells were stained with the 4H1 anti-GMRα monoclonal antibody and an anti-mouse-PE secondary antibody and the GM-CSF receptor-positive cells were purified by FACS. Purified cells were then plated in either GM-CSF or control cocktail for 24 hours with a BrdU pulse for the last 4 hours. Cells were then fixed and analysed for BrdU incorporation by flow cytometry. As shown in Figure 4, GM-CSF is able to promote BrdU incorporation in cells transduced with the wtβc, βcTyr577Phe, βcSer585Gly, βcTyr577Phe/Ser585Gly, βcF7 but not the βcF8 mutant. Thus, βc tyrosine phosphorylation in response to GM-CSF appears to have an important role in promoting cell proliferation. Furthermore, the results indicate that GM-CSF is able to independently regulate cell proliferation and survival in primary hemopoietic cells and that these biological responses are regulated by distinct motifs in the βc with the βcTyr577Phe/Ser585Gly mutant being unable to regulate cell survival but is able to regulate cell proliferation and the converse being true for the βcF8 mutant which is able to promote cell survival but unable to regulate cell proliferation.

Example 5: Ability of the wt and mutant βc to transduce signals through the JAK/STAT, the PI 3-kinase and Ras/Map kinase pathways

(a) Immunoblotting

Transduced fetal liver cells were factor-deprived for 12 hours in IMDM containing 0.5% HI FCS and then stimulated with 50ng/ml human GM-CSF. Cells were lysed in RIPA buffer (150mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% sodium dodecylsulphate (SDS), 50mM Tris-HCl, pH 7.4). Cell lysates were then subjected to SDS polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis using standard conditions and signals were developed using enhanced chemiluminescence (ECL)(Amersham Pharmacia or West Dura from Pierce).

GM-CSF is able to regulate intracellular signalling via the JAK/STAT, the PI 3-kinase and the Ras/Map kinase pathways. To examine the ability of the wt and mutant βc to transduce signals through these pathways a series of Western blots were performed using phospho-specific antibodies to JAK2, STAT5, Akt, ERK Fetal liver cells transduced with the wt βc exhibited increased phosphorylation of JAK2, STAT5, Akt, ERK in response to GM-CSF (Figure 5). Similarly, cells expressing the βcTyr577Phe, βcSer585Gly, and βcF7 mutants also demonstrated phosphorylation of these signalling molecules. While cells expressing the βcF8 mutant were able to induce phosphorylation of JAK2 and STAT5 in response to GM-CSF, the phosphorylation of Akt and, ERK was decreased compared to that observed in cells expressing the wt βc. The βcTyr577Phe/Ser585Gly mutant, which was unable to promote cell survival in reponse to GM-CSF, was able to regulate the phosphorylation of JAK2, STAT5, Akt, ERK {and GSK-3b} in a similar manner to that observed for cells expressing the wt βc. Thus the inability of GM-CSF to promote cell survival in fetal liver cells transduced with the βcTyr577Phe/Ser585Gly mutant was not due to an inability of this mutant receptor to regulate the activation of either JAK2, STAT5, Akt, or ERK

Example 6: Interactions of the Shc and 14-3-3 to Tyr577 and Ser585 respectively

To study the possible interaction of Shc , which is known to bind to Tyr 577, and 14-3-3 which we found to bind to Ser 585, we stimulated cells with GM-CSF and measured their association. We found that GM-CSF stimulation caused tyrosine phosphorylation of 14-3-3 and that Tyr 179 was necessary for 14-3-3 interacting with Shc (Figure 5). Furthermore Tyr 179 of 14-3-3 was also necessary for PI-3 kinase activation (Figure 6) and AKT activation (Figure 7) in response to GM-CSF.

The possibility that the bidentate motif or parts of it could be intrinsically activated in leukaemia was also searched. Importantly we found in primary cells from CML or AML patients that intrinsic activation of Ser 585 was taking place

inboth cases (Figure 8) indicating that this motif and its abnormal activation may play a role in disease.

Example 7: Identification of similar potential bidentate motifs in other receptors.

The identification of a novel phosphotyrosine/phosphoserine bidentate motif that is important in regulating cell survival in these studies prompted us to examine whether other cell surface receptors may also contain similar motifs. Phosphorylated Tyr577 of βc is known to bind Shc via its PTB domain whereas phosphorylated Ser585 is known to bind 14-3-3. We therefore scanned the cytoplasmic domains of cell surface receptors for a PTB binding site followed by a 14-3-3 binding site using software available to the skilled addressee . The PTB domain of Shc recognizes a N-X-X-Y motif (where Y is phosphorylated). 14-3-3 was originally demonstrated to binding two possible motifs; a mode 1 site (R-S-X-S/T-X-P) or a mode 2 site (R-X/Ψ-X/Ψ-X/Ψ-S/T-X-P)(where S or T is phosphorylated and Ψ is a hydrophobic residue). Variations on these prototypic 14-3-3 binding motifs have since been reported with K, H or Q also being tolerated at the -3 and -4 positions relative to the phosphoserine/phosphothreonine. In addition, the proline at the +2 position, which has been reported to be important for the correct exit of the bound protein from the binding groove of 14-3-3, has been found to be dispensible if the 14-3-3 binding motif occurs close to the C-terminus of a protein. Searching for motifs that allow these variations, we have identified conserved putative bidentate tyrosine/serine motifs in a range of cell surface receptors (Table 1). In addition to the noteable prevalence of such a bidentate motif in cell surface receptors, it is also striking that in some cases this motif appears to be conserved within specific members of receptor families such as the FGF, LDL and integin receptor families. Alignment of these motifs suggests a putative consensus bidentate motif, N-X-X-Y-$(X)_{1-13}$-[R/K/H/Q]-$[X/\Psi]_{2-3}$-S/T-X-P (where X is any residue, Y is phosphotyrosine, S/T is phosphoserine or phosphothreonine and Ψ is a hydrophobic residue). We also considered the possibility that receptors may also utilize alternative motifs in which the tyrosine residue was not part of a PTB binding site but rather an SH2 binding site. Searching for an adjacent

tyrosine residue/14-3-3 binding site, we identified alternative putative bidentate motifs in a range of cell surface receptors. Alignment of these motifs gave the consensus $\underline{Y}$-$(X)_{1-16}$-[R/K/H/Q]-$[X/\Psi]_{2-3}$-$\underline{S}/\underline{T}$-X-P. Our finding that the Tyr577/Ser585 bidentate motif is important in regulating cell survival in response to GM-CSF and that similar motifs are also found in other cell surface receptors suggests that this novel motif may play a fundamental role in regulating intracellular signalling in response to a wide range of cytokines and growth factors.

Table 1

PILE UP:

betaR	NGPYLG.....PP..HSRSLP
Acetylcholine R (ISOFROM?)	NVHYRT.....P...KTHTMP
Acetylcholine R alpha-5 (CONSERV?)	**RYFTQKEE.....TESGSGP
C-C chemokine receptor 6	NKKYELQDRDVCE....P.RYRSVSEP
Middle T antigen	NPTYSVM.....RSHSYP
integrin alpha 1	NIFYLIR...KSGSFPMPELKLSISFP
FGFR2 (KGF R)	NEEYLDLSQ.....PLEQYSPSYP
FGFR1 (flg)	NQEYLDLSM.....PLDQYSPSFP
FGFR5	NATYKVD.....VIQRTRSKP
Erb4	NPEY.....HSASSGP
Erb4 (second)	NPDY.....WNHSLP
Vaccinia virus protein A36R	NPSYSSNPFVNYN....KTSICSKSNP
Macrophage mannose R (MRC1)	NTLY.....FNSQSSP
LDLR	NPVYQKTTEDEVHI...CHNQDGYSYP
VLDL (rat)	NPVYLKTTEEDLSIDIG..RH.SASVG (near end of protein)

LRP1 low density lipoprotein receptor-related protein 1
NPTYKMYEGGEPDDVGGLLDADFALDPDKPTNFTNPVY

integrin beta 1	NPIY.....KSAVTTVV (end of protein)
interin beta 7	NPLY.....KSAITTTV (end of protein)
integrin beta 3	NPLY.....KEATSTFT (end of protein)
integrin beta 5	NPLY.....RKPISTHT (end of protein)
integrin beta 6	NPLY.....RGSTSTFK

G-CSFR1 (second)	PGHYL.....RCDSTQP
G-CSFR1	VQTYVLQ.....GDPRAVSTQP

```
g-csf-r                                  QVLYGQLL.............GSPTSP
(CHECK?)
IL-6B (gp130)                            HSGYRHQVPSVQVF.....SRSESTQP

leptinR.                                 WKMYEVYDA.........KS.KSVSLP
prolactinR...                            KIPYFHA..........GGS.KCSTWP
insulinR                                 ELDYCLKGLKL.......P.S.RTWSPP
irs-1 ...                                SGDYMPM............SPKSVSAP
IGFI R
      SFYYSEENKLPEPEELDLEPENMESVP(LDPSASSSSLP)1283=survl.
flt3 R                                   EEIYIIM.....QSCWAFDSRKRPSFP
VEGFR2 (FLK1)                            ISQYLQN............S.KRKSRP
PDGF R-alpha                             GTAY................GLSRSQP
IL-9R              ***YLPQEDWAP.........TSLTRP (CONSERV?)
p75 NTR
      LVAYIAFKRWNSCKQN...KQGANSRPVNQTPPPEGEKLHSDSGIS(phosphorylated)
```

MOTIF (forward) **n-X-X-Y-X(3,17)-[RKHQ]-X(2,3)-[ST]-X-P**

```
EGFR               RYSSDPTGALTEDSIDDTFLPVPEYINQSVPKRPAGSVQNPVY.. (NPEY)
Erb2               KTLSPGKNGVVKDVFTF.................GGAVENPEY
Voltage-depend     RTHSLP.................................NDSY
T-type Ca chan.
alpha-1G subunit

EPO R              SDGPYSNPYENSLIPAAEPLPPSYVACS (Y NB in PI 3-K; S is
end of protein, JBC 270: 23402)
```

MOTIF (reverse) **[RKHQ]-X(2,3)-[ST]-X-P-X(0,33)-N-X-X-Y**

```
TRHR receptor                                                  HFSTELD
IL-2R beta       NQGYFFFHLPDALEIEACQVYFTYDPYSEEDPDEGVAGAPTGSSP
```

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

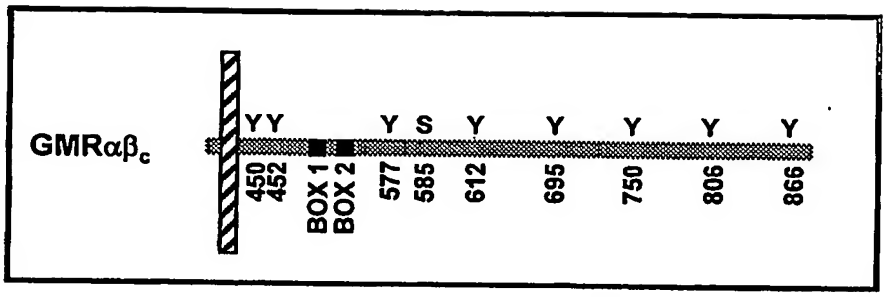
PHILLIPS ORMONDE & FITZPATRICK

Attorneys for:

MEDVET SCIENCE PTY LTD

David P Fitzpatrick

Figure 1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Y S Y Y Y Y Y
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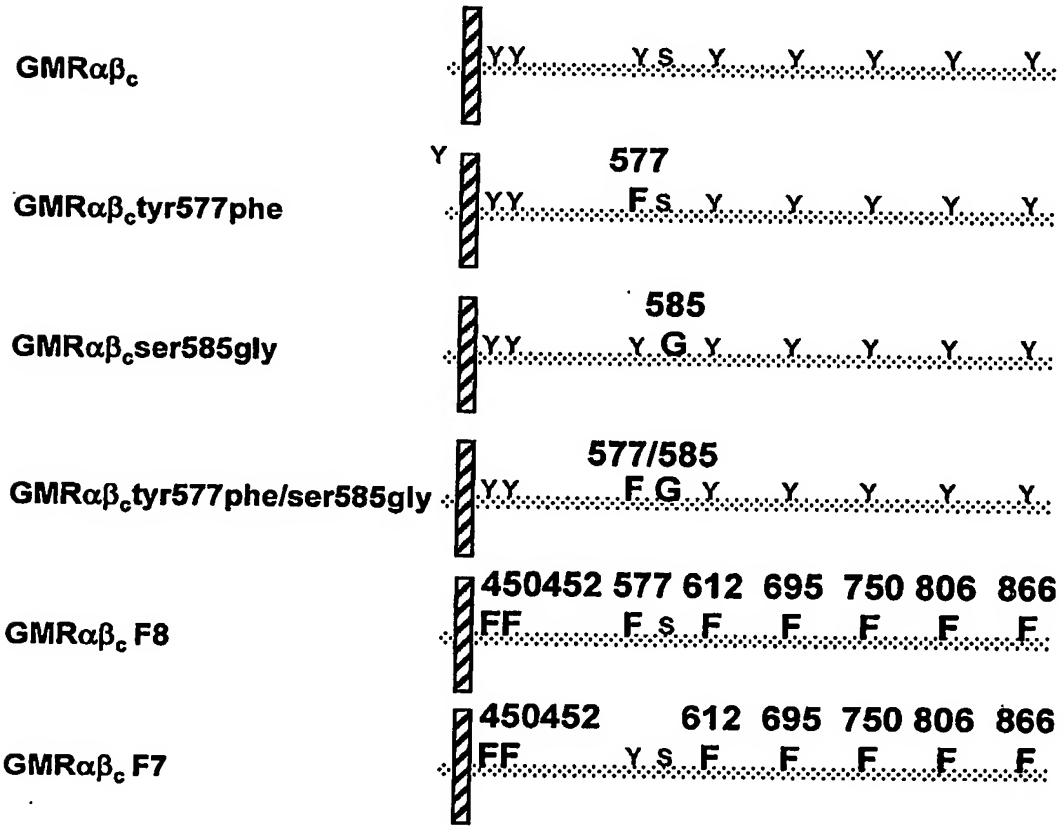
452
BOX 1
BOX 2
577
585
612
695
750
806
866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GMRαβctyr577phe
577
GMRαβcser585gly
585
GMRαβctyr577phe/ser585gly
577/585
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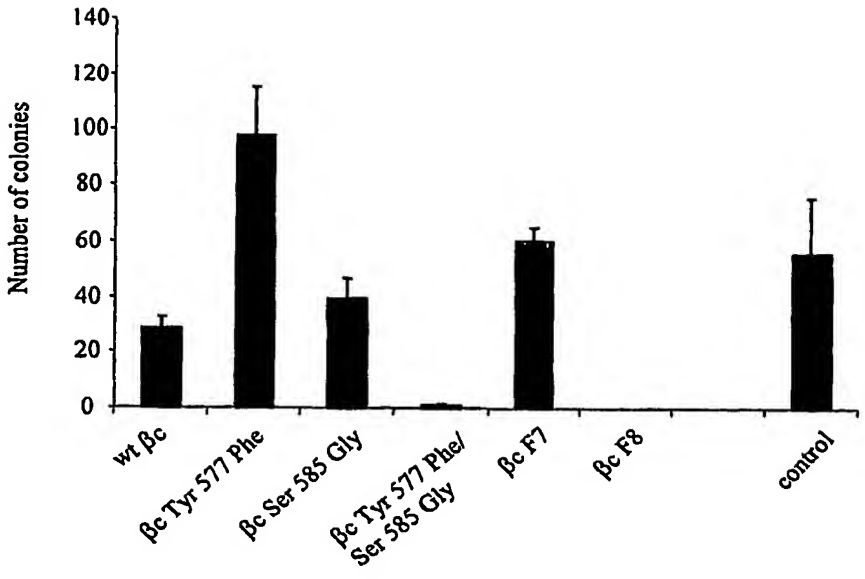
450452 577 612 695 750 806 866
GMRαβc F7
450452 612 695 750 806 866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140
120
100
80
60
40
20
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wt βc
βc Tyr 577 Phe
βc Ser 585 Gly
βc Tyr 577 Phe/ Ser 585 Gly
βc F7
βc F8
control

Figure 2

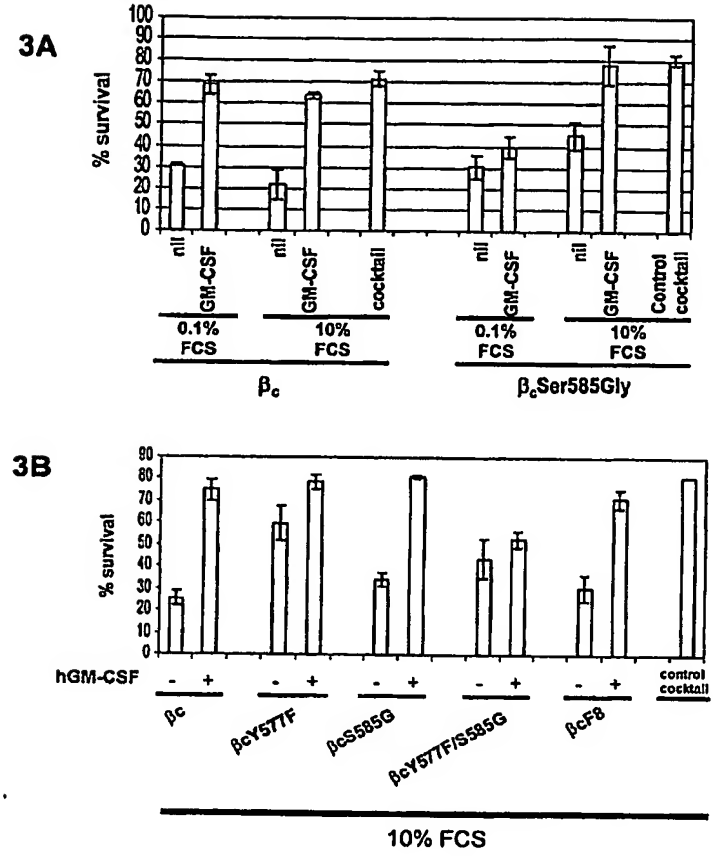
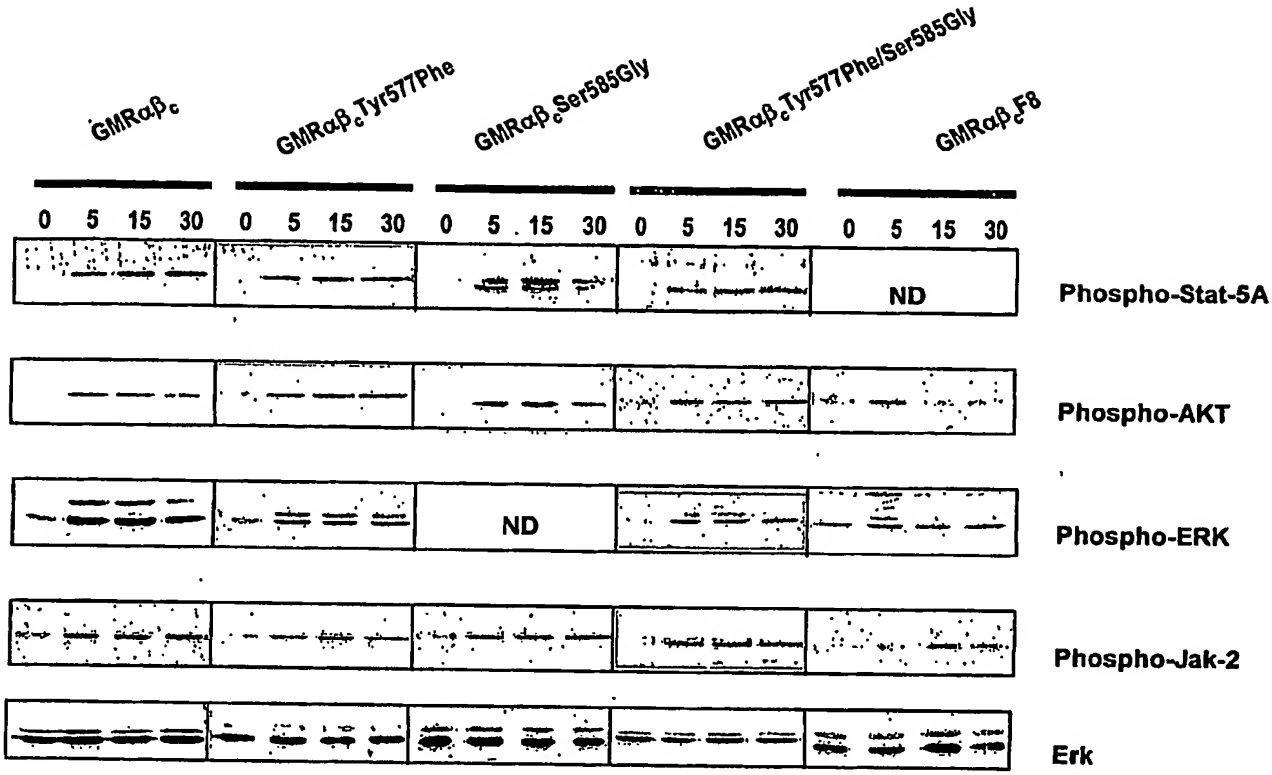

3A
% survival
nil
GM-CSF
nil
GM-CSF
cocktail
nil
GM-CSF
nil
GM-CSF
Control cocktail
0.1% FCS
10% FCS
0.1% FCS
10% FCS
βc
βcSer585Gly
3B
% survival
hGM-CSF
- + - + - + - + - +
control cocktail
βc
βcY577F
βcS585G
βcY577F/S585G
βcF8
10% FCS

Figure 3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GMRαβcSer585Gly
GMRαβcTyr577Phe/Ser585Gly
GMRαβcF8
0 5 15 30 0 5 15 30 0 5 15 30 0 5 15 30 0 5 15 30
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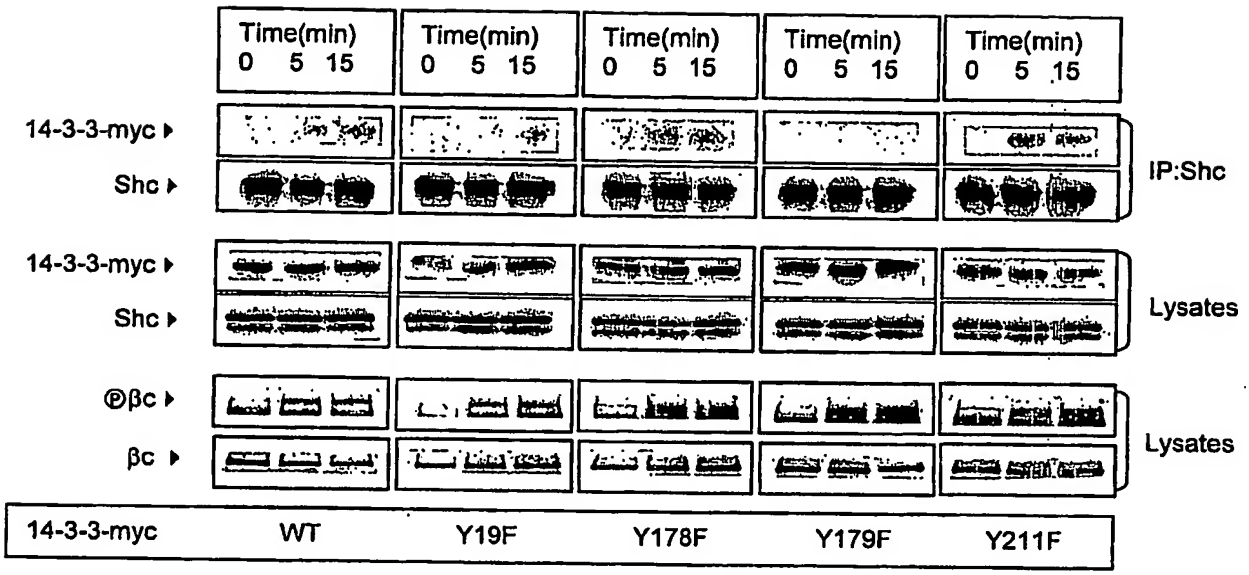
Phospho-Stat-5A
Phospho-AKT
ND
Phospho-ERK
Phospho-Jak-2
Erk

Figure 4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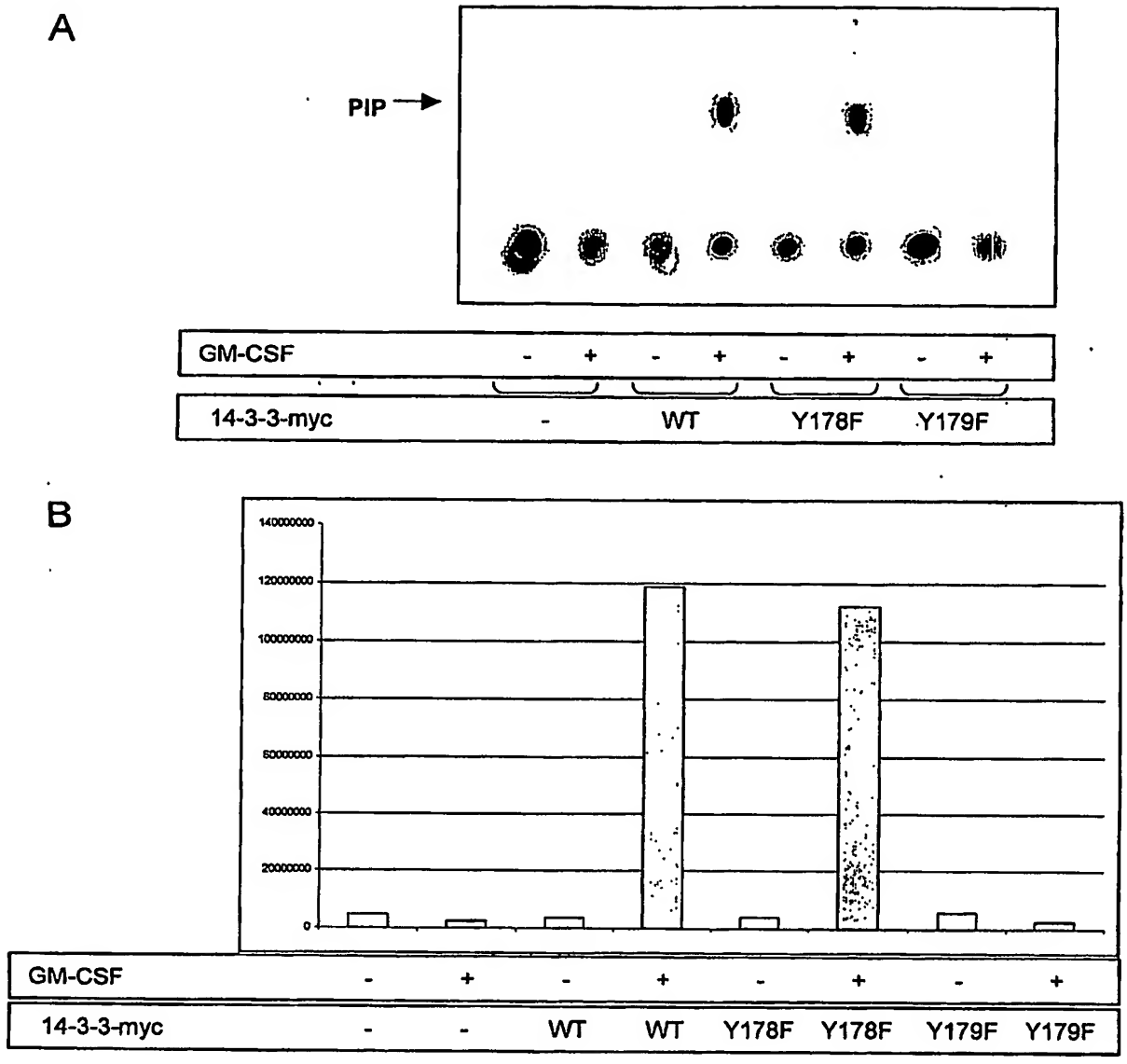
Time(min)
0 5 15
Time(min)
0 5 15
Time(min)
0 5 15
Time(min)
0 5 15
14-3-3-myc
Shc
IP:Shc
14-3-3-myc
Shc
Lysates
Ⓟβc
βc
Lysates
14-3-3-myc
WT
Y19F
Y178F
Y179F
Y211F

Figure 5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PIP
GM-CSF
- + - + - + - +
14-3-3-myc
- WT Y178F Y179F
B
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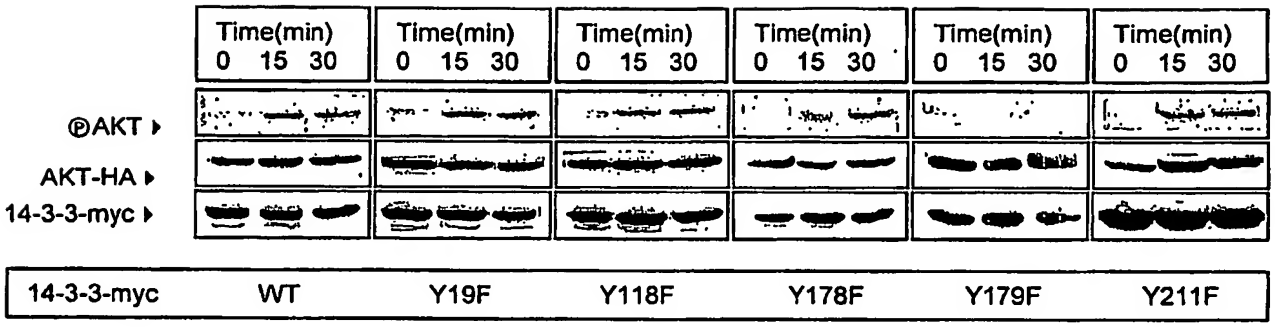
120000000
100000000
80000000
60000000
40000000
20000000
0
GM-CSF
- + - + - + - +
14-3-3-myc
- - WT WT Y178F Y178F Y179F Y179F

Figure 6

A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0 15 30
Time(min)
0 15 30
Time(min)
0 15 30
Time(min)
0 15 30
Time(min)
0 15 30
Time(min)
0 15 30
ⓅAKT
AKT-HA
14-3-3-myc
14-3-3-myc WT Y19F Y118F Y178F Y179F Y211F

B

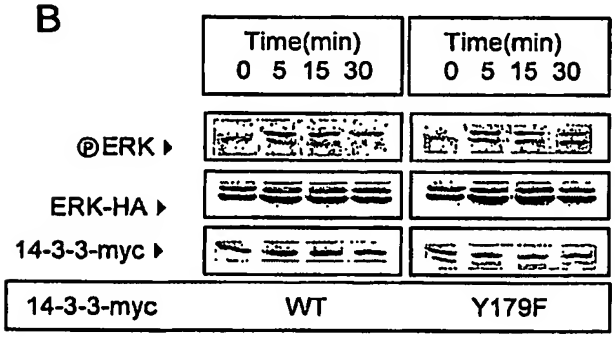
Time(min)
0 5 15 30
Time(min)
0 5 15 30
ⓅERK
ERK-HA
14-3-3-myc
14-3-3-myc WT Y179F

Figure 7

Figure 8

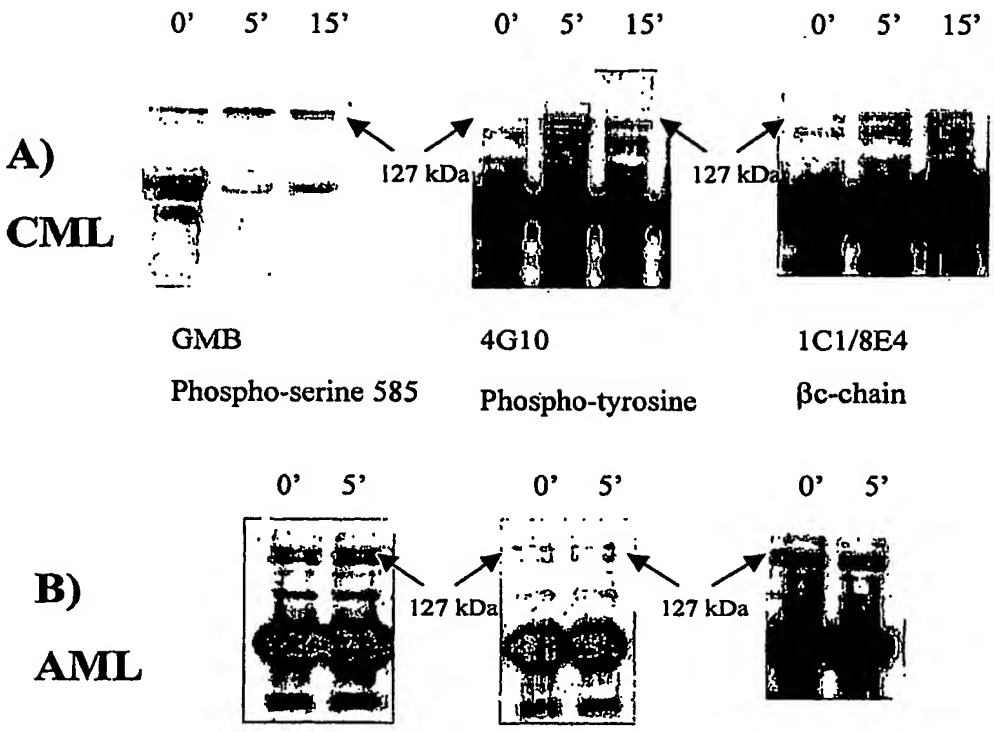

Figure 9

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     101  QSFVVTDVDY FSFQPDRPLG TRLTVTLTQH VQPPEPRDLQ ISTDQDHFLL
     151  TWSVALGSPQ SHWLSPGDLE FEVVYKRLQD SWEDAAILLS NTSQATLGPE
     201  HLMPSSTYVA RVRTRLAPGS RLSGRPSKWS PEVCWDSQPG DEAQPQNLEC
     251  FFDGAAVLSC SWEVRKEVAS SVSFGLFYKP SPDAGEEECS PVLREGLGSL
     301  HTRHHCQIPV PDPATHGQYI VSVQPRRAEK HIKSSVNIQM APPSLNVTKD
     351  GDSYSLRWET MKMRYEHIDH TFEIQYRKDT ATWKDSKTET LQNAHSMALP
     401  ALEPSTRYWA RVRVRTSRTG YNGIWSEWSE ARSWDTESVL PMWVLALIVI
     451  FLTIAVLLAL RFCGIYGYRL RRKWEEKIPN PSKSHLFQNG SAELWPPGSM
     501  SAFTSGSPPH QGPWGSRFPE LEGVFPVGFG DSEVSPLTIE DPKHVCDPPS
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     601  SLPDILGQPE PPQEGGSQKS PPPGSLEYLC LPAGGQVQLV PLAQAMGPGQ
     651  AVEVERRPSQ GAAGSPSLES GGGPAPPALG PRVGGQDQKD SPVAIPMSSG
     701  DTEDPGVASG YVSSADLVFT PNSGASSVSL VPSLGLPSDQ TPSLCPGLAS
     751  GPPGAPGPVK SGFEGYVELP PIEGRSPRSP RNNPVPPEAK SPVLNPGERP
     801  ADVSPTSPQP EGLLVLQQVG DYCFLPGLGP GPLSLRSKPS SPGPGPEIKN
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